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MATURATION OF SPERMATOZOA IN THE RABBIT EPIDIDYMIS : FERTILIZING ABILITY AND EMBRYONIC MORTALITY IN DOES INSEMINATED WITH EPIDIDYMAL SPERMATOZOA

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SOMMAIRE

Les spermatozoïdes subissent au cours de leur passage dans l'épididyme des modifications morphologiques et physiologiques, dont la plus importante est probablement l'acquisition du pouvoir fécondant. Chez le Lapin, la région de l'épididyme où les spermatozoïdes deviennent fécondants est bien localisée : c'est la partie distale du corps de l'épididyme. Un œuf seulement sur 93 a été fertilisé par des spermatozoïdes de la tête et du corps proximal (CAP + COR 1) de l'épididyme, 57 p. 100 de 142 œufs et 95 p. 100 de 162 œufs l'ont été après insémination de spermatozoïdes soit du corps distal soit de la partie distale de la queue de l'épididyme. En comparant la morphologie (position de la gouttelette cytoplasmique) des spermatozoïdes de la partie proximale et distale du corps de l'épididyme, il n'a pas été possible d'établir une corrélation entre la perte de la gouttelette cytoplasmique et le degré de maturité du spermatozoïde. L'étude de la motilité des spermatozoïdes s'est révélée tout aussi décevante. Bien que la plupart des spermatozoïdes de la tête de l'épididyme aient une motilité tout à fait différente de celle des spermatozoïdes de la queue (décrivant des cercles au lieu de progresser en avant), ceci ne les empêche pas de franchir la jonction utéro-tubaire et d'être retrouvés dans l'oviducte aussi nombreux qu'après accouplement.

Lorsque les lapines sont inséminées avec des spermatozoïdes épididymaires, peu d'embryons arrivent à terme : 77,6 et 65,4 p. 100 des œufs fécondés par des spermatozoïdes du corps distal ou de la queue de l'épididyme sont perdus au cours de la gestation. Les pertes s'élèvent à 38,9 p. 100 quand des spermatozoïdes éjaculés sont inséminés de la même manière directement dans l'utérus. Plus d'embryons dégénèrent avant l'implantation qu'après. Les principales anomalies relevées durant la première partie de la gestation sont : 1) 9,28 p. 100 de fécondations anormales, les œufs possédant trois ou quatre pronuclei ; 2) un retard dans le développement des œufs fécondés par les spermatozoïdes épididymaires. La possibilité, que l'une ou l'autre de ces anomalies puissent être responsables de la mortalité embryonnaire observée, est discutée.

INTRODUCTION

In mammals, spermatozoa do not possess their full fertilizing ability upon leaving the testis. During their passage through the epididymis, a progressive maturation occurs and they show an increasing capacity for fertility (YOUNG, 1931) ; NISHI-

KAWA and WAIDE, 1952 ; BLANDAU and RUMERY, 1964 ; BEDFORD, 1966). This functional maturation is concomitant with morphological as well as physiological and biochemical changes. The most marked of the morphological changes are in the position of the cytoplasmic droplet (LAGERLOF, 1934 ; BRANTON and SALISBURY, 1947 ; NICANDER, 1957), size, shape and internal structure of the acrosome (FAWCET and HOLLENBERG, 1963 ; BEDFORD, 1963-1965), structural organization of the mitochondria of the sperm middle piece (Anberg, 1957), membrane permeability (ORTAVANT, 1953 ; GLOVER, 1962 ; AMANN and ALMQUIST, 1962), and percentage of abnormal spermatozoa (GLOVER, 1962 ; AMANN and ALMQUIST, 1962). Capacity for motility increases as the spermatozoa pass through the epididymis (TOURNADE, 1913 ; YOCHEM, 1930), and a progressive loss of water occurs as well as a corresponding increase in the specific gravity and the light-reflection power of sperm (LINDAHL and KIHLSTROM, 1952). A greater resistance to cold shock (LASLEY and BOGART, 1944 ; BIALY and SMITH, 1959 ; WHITE and WALES, 1961), and to variations in pH (YOCHEM, 1930) has been observed as well as possible distinct metabolic characteristics between epididymal and ejaculated spermatozoa (HENLE and ZITTLE, 1942 ; LARDY and GOSH, 1952 ; WHITE and WALES, 1961 ; SALISBURY and LODGE, 1962). It seems probable that the acquisition of the fertilizing capacity during this complex maturation process results from one or several of these morphological or biochemical changes. BLANDAU and RUMERY, 1964, attribute the absence of fertilizing ability of spermatozoa from the *caput epididymidis* in the rat to their non-progressive motility pattern prohibiting them from going through the utero-tubal junction. The purpose of the present investigation was to study the morphology of spermatozoa from different epididymal segments their ability to go through the utero-tubal junction, their fertilizing capacity, and the development of ova thus fertilized.

MATERIAL AND METHODS

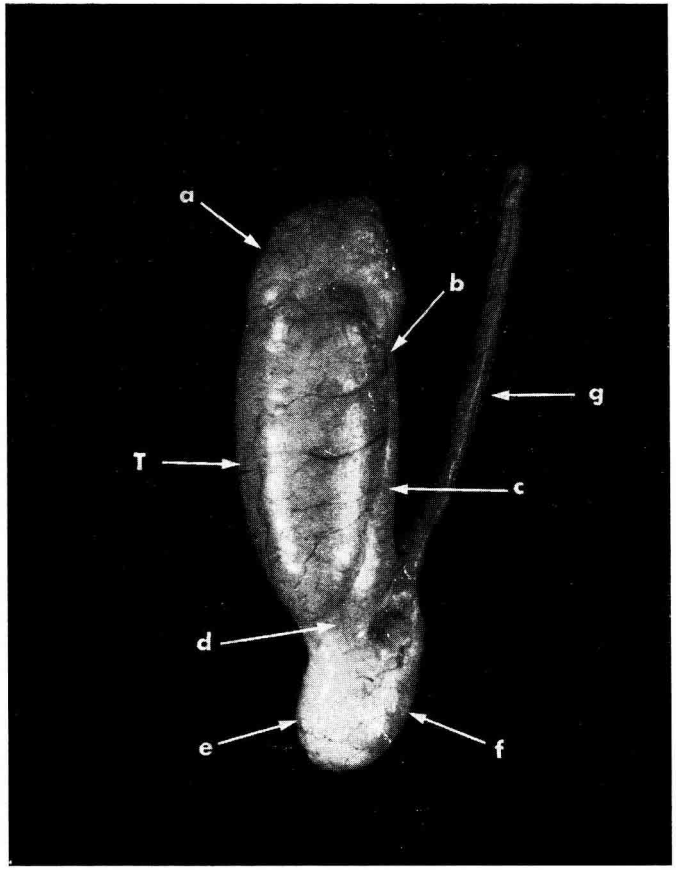
The rabbits used all through this study were phenotypically *White New Zealand.*

Preparation of sperm suspension

Thirty-four males, 9-12 months old, weighing 3.6 ± 0,05 kg were used. They were at sexual rest for at least three weeks before they were killed by an overdose of Diabutal (Diamond Laboratory). The reproductive system was removed quickly and was immersed immediately in 0.9 p. 100 NaCl. Each epididymis was separated from the testis. Fat and blood vessels were removed and the epididymis was dissected as shown in Plate 1. Special care was exercised to separate the *proximal* from the *distal cauda* without contamination : the middle line of cleavage formed by the connective tissue was used as a guide. Each segment was cut starting from the *caput epididymidis* toward the *cauda* and a clean set of forceps and scissors was used for cutting each section (this was done all through the experiment by the same operator). Each segment was thoroughly washed in a beaker containing 5 ml of 0.9 p. 100 NaCl and then placed in a depression slide containing 1 ml of 0,9 p. 100 NaCl. Using iridectomy scissors, the tissue was cut in fine pieces, thus releasing the spermatozoa into the medium ; the pieces of tissue were then removed.

The following abbreviations will be used for the different parts of the epididymis (see Plate 1) :

Caput epididymidis	: CAP
Upper *corpus epididymidis*	: COR 1
Lower *corpus epididymidis*	: COR 2
Upper *proximal cauda epididymis*	: CAU 1
Proximal *cauda epididymidis*	: CAU 2
Distal *cauda epididimidymis*	: CAU 3



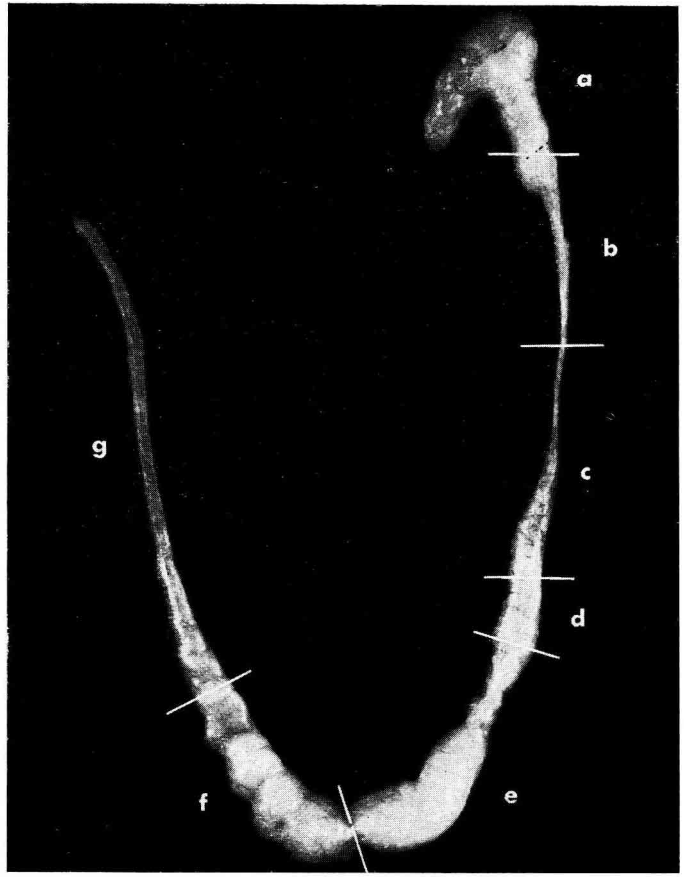


PLATE. 1. — *Photographs of the intact testis and epididymis of the rabbit and of the dissected epididymis. The latter was dissected apart along natural plane of cleavage into seven segments distinguishable on the basis of tubule diameter and convolutional pattern.* (T = testis : a = *caput epididymidis :* b = upper *corpus epididymidis :* c = lower *corpus epididymidis :* d = upper *proximal cauda epididymidis :* e = proximal *cauda epididymidis :* f = distal *cauda epididymidis :* g = *ductus deferens*).

PLANCHE. 1. — *Photographies de testicule et épididyme du lapin et de l'épididyme disséqué. Ce dernier a été disséqué selon les plans de clivage naturels, en plusieurs segments différenciés par le diamètre et les convolutions du canal épididymaire.*

In a first series of experiments, sperm suspensions from CAP, CAU 2 and CAU 3 were inseminated in three different females. In a second series, spermatozoa from COR 1, COR 2 and CAU 3 were used. In a third series, spermatozoa from COR 2 and CAU 3 were used. In each series, sperm suspensions, were counted with an hæmocytometer and the suspension was adjusted in order to have in each group of three (or two) females, a like number of spermatozoa inseminated.

When ejaculated sperm suspensions were used, males, at sexual rest at least one week prior to service, were collected with an artificial vagina. Sperm concentration was determined and the semen was diluted with 0.9 p. 100 NaCl in which 2 000 i. u. Penicillin G/ml was added. The final sperm concentration in all cases was 20×10^3/ml.

Sperm morphology

A drop from each sperm suspension was mixed on a slide with a drop of 0.9 p. 100 NaCl, to which 1 p. 100 formalin was added in order to immobilize the spermatozoa. The preparation, without any prior smearing, was sealed under a coverslip with a vaseline-paraffin mixture. The delay between death and the sample preparation was approximately 30 minutes. Two slides for each sperm suspension were prepared and read by two different observers. Slides were scanned under a phase contrast microscope. Two longitudinal axes only were traversed. For each epididymal region the morphology of 1 000 consecutive spermatozoa was studied under a magnification of $\times$ 512.

Preparation of the female for artificial insemination

Eithty-one sexually mature virgin does were used. The age range was 6-8 months old. Each doe was caged individually for 21 days before being used in an experiment. Ovulation was induced by an intravenous injection of 50 i. u. of chorionic gonadotropin (HCG) from Ayerst Laboratory (A. P. L.). One hour later the does were anesthetized with Diabutal, opened through a mid-line incision, and both uterine horns were exteriorized. The sperm suspension, drawn in a 1 ml syringe fitted with a No 23 hypodermic needle, was then injected, 0.3 ml in each horn. Insemination was done 1 hour 15 minutes after injection of HCG.

In a control series, 35 females were mated with two bucks. These females had been injected with HCG 1 hour 15 minutes previously to ensure against failure of ovulation and to simulate the conditions of the experimental series.

Fertilization

Each female was killed 24 to 29 hours after injection of HCG. The oviducts were removed and flushed from the uterine end toward the ovarian end with 2 ml of 0.9 p. 100 NaCl. Ova were mounted between a glass slide and a coverslip supported by four dots of a vaseline-paraffin mixture and examined with the ordinary and phase contrast microscope for evidence of fertilization. The criterion for fertilization was the presence of both pronuclei or of cleavage.

Number of spermatozoa within the eggs

After the evidence of fertilization was obtained, the coverslip was pressed down in order to squash the ova, and the total number of spermatozoa in each ovum was determined with an $\times$ 40 objective. Spermatozoa trapped in the mucin layer were not included in the total figure given.

Determination of the total number of sperm present in the oviduct

After the ova were removed, the flushings were transferred into a 5 ml centrifuge tube. All the glassware used throughout this experiment was thoroughly washed to avoid contamination and then siliconed. Five hæmocytometers were prepared, and the suspension was mixed for one minute on a Vortex mixer before filling each chamber on each hæmocytometer. All the spermatozoa present in the central square millimeter were counted, thus giving ten determinations for each suspension.

Suspensions were then centrifuged for ten minutes in an Adams serofuge at 1 000 *g* The supernatant was poured out, leaving approximately 0.15 ml in the tube. Suspensions were thoroughly mixed as described above and two hæmocytometers were prepared. With this method the number of spermatozoa counted was usually smaller than that obtained with the first method and variations were much greater ; it was used only to ascertain the presence of spermatozoa in the flushings. The number of spermatozoa given in Table 1 corresponds to the first method outlined above.

Prenatal mortality

After intrauterine insemination of either epididymal or ejaculated spermatozoa, 31 females were allowed to continue through pregnancy. Laparotomy, under Diabutal anesthesia, was performen ten days after insemination. The number of *corpora lutea* in each ovary and the number of implantation sites in each uterine horn were counted. The external diameter of the uterine swellings at right angles to the long axis of the uterus was measured with vernier callipers.

After recovery, the does were allowed to continue their gestation until day 28, when they were autopsied. The number of *corpora lutea*, resorption sites and the number and weight of live and dead fœtuses were recorded.

RESULTS

Sperm morphology

The distribution of the cytoplasmic droplet between the populations of spermatozoa from the six epididymal segments studied is shown in table 1. Cytoplasmic droplets are located on the neck of 58 p. 100 of the spermatozoa from the *caput* region. In the *corpus epididymidis*, half of the spermatozoa have droplets on the middle piece or have no droplets at all. Sixty percent of the spermatozoa from CAU 2 and CAU 3 appeared without droplets. « No-droplet » spermatozoa are present in significant numbers in the *caput* (17.6 p. 100 and the *corpus* (43.5 p. 100). On the other hand, « neck-droplet » spermatozoa can still be found in CAU 3 (6.2 p. 100). It thus seems reasonable to assume that, for most spermatozoa, the migration of the cytoplasmic droplet away from the neck occurs in the distal *caput* and that the migration down the middle piece takes place during the passage through the *corpus*. This pattern holds true for the majority of spermatozoa, but « no-droplet », « middle piece-droplet » and « neck-droplet » spermatozoa can be found in significant numbers at all levels of the epididymal duct. Disintegrated spermatozoa are found in every segment of the epididymis. The CAU 3 has a higher percentage of disintegrated spermatozoa than either the caput ($P < 0.05$) or COR 1 ($P < 0.01$). The difference between CAU 3 and COR 2 is at the limit of significance ($0.05 < P < 0.1$). There is no difference between the segments of the *cauda* (1, 2 or 3). The mean percentages of coiled spermatozoa are not statistically different in any of the regions studied. A spermatozoon was considered coiled when at least one complete loop was observed in the tail.

Passage of epididymal spermatozoa through the utero-tubal junction

Mean numbers of spermatozoa found in the oviduct and per fertilized ovum after uterine insemination of either 2.7, 9.0 or 15.1 $\times$ 10^6 epididymal spermatozoa, or after double mating, are shown in table 2. After normal mating, 9 800 $\pm$ 3 300 spermatozoa are found in the oviduct 24 hours after insemination, 3 500 $\pm$ 900 after 25-26 hours, and 4 100 $\pm$ 600 after 27-28 hours. There are no statistically significant differences between these three groups, therefore they have been averaged together in table 2. Spermatozoa from the *caput epididymidis* are able to migrate through

TABLE I

Morphology of epididymal spermatozoa in the rabbit

TABLEAU I

Morphologie des spermatozoïdes de l'épididyme chez le lapin

Epididymal Segment	« Neck-droplet » spermatozoa (%)	« Middle piece-droplet » spermatozoa (%)	« No-droplet » spermatozoa (%)	Abnormal spermatozoa (%)	Disintegrated spermatozoa (%)	Coiled spermatozoa (%)
Caput epididymidis	58.0 ± 2.9*	23.9 ± 1.4	17.6 ± 1.2	2.2 ± 0.6	2.5 ± 0.3	1.5 ± 0.3
Upper *corpus epididymidis*	10.3 ± 2.2	47.0 ± 2.7	42.5 ± 3.6	2.8 ± 0.5	1.2 ± 0.2	0.9 ± 0.2
Lower *corpus epididymidis*	8.0 ± 1.8	48.3 ± 3.4	43.5 ± 3.6	2.1 ± 0.5	2.3 ± 0.4	0.9 ± 0.2
Upper proximal *cauda epididymidis*	6.5 ± 2.2	42.3 ± 4.0	50.9 ± 5.3	3.5 ± 0.7	2.9 ± 0.5	1.5 ± 0.3
Proximal *cauda epididymidis*	5.3 ± 1.1	28.9 ± 3.3	65.5 ± 3.7	1.9 ± 0.5	2.9 ± 0.5	2.1 ± 0.4
Distal *cauda epididymidis*	6.2 ± 1.3	37.2 ± 3.0	55.6 ± 3.6	1.4 ± 0.1	5.3 ± 0.8	2.0 ± 0.4

* Standard error.

the utero-tubal junction, and there is no significant difference between their number recovered from the oviduct and the number recovered after mating. However when a same number of spermatozoa (15.1×10^6) from CAU 2 or CAU 3 is inseminated, more spermatozoa are recovered from the oviduct ($P < 0.01$), indicating that the ascent of the *caput* spermatozoa is somewhat impaired. There is no significant difference in the number of spermatozoa from COR 1, COR 2 or CAU 3 recovered from the oviduct after intrauterine insemination. Likewise, there is no significant difference in the number of spermatozoa within or inside the *zona pellucida* when spermatozoa from the COR 2 or the CAU 3 are inseminated.

TABLE 2

Total number of spermatozoa per fertilized ovum and of spermatozoa recovered from the oviduct 24 to 28 hours after intrauterine insemination of epididymal spermatozoa or after double mating

TABLEAU 2

Nombre total de spermatozoïdes par œuf fécondé et par oviducte 24 à 28 heures après insémination intrautérine avec des spermatozoïdes de l'oviducte l'épididyme ou après double accouplement.

Conditions of insemination	Total number of spermatozoa inseminated ($\times 10^6$)	Total number of spermatozoa $\times 10^3$ recovered from the oviduct	Total number of spermatozoa per fertilized ovum	Percentage of fertilized ova
Mating	?	5.6 ± 1.2		95.4
Sperm suspension from : *Caput epididymidis*	15.1 ± 1.7*	3.9 ± 1.2	1	2.1
Upper *corpus epididymidis*	2.7 ± 0.7	5.9 ± 3.0	—	0
Lower *corpus epididymidis*	2.7 ± 0.7	4.2 ± 1.3	8.7 ± 2.2	63.6
	9.0 ± 0.5	2.5 ± 0.7	9.0 ± 1.5	49.3
Upper proximal *cauda epididymidis*	15.1 ± 0.5	6.0 ± 1.6	17.2 ± 3.0	73.6
Proximal *cauda epididymidis*	15.1 ± 1.7	17.0 ± 3.8	24.6 ± 2.1	92.7
Distal *cauda epididymidis*	2.7 ± 0.7	2.8 ± 0.7	5.4 ± 1.3	84.0
	9.0 ± 0.5	7.0 ± 3.2	21.5 ± 2.9	95.5
	15.1 ± 1.7	29.2 ± 8.1	28.0 ± 2.3	94.5

* Standard error.

Fertilizing ability

Results are summarized in table 3. Spermatozoa from the *caput* and the COR 1 are unable to fertilize. Lower than the middle *corpus*, they do attain their fertilizing ability, but the percentage of fertilized ova from the COR 2

spermatozoa is lower (57.0 p. 100) than the one from the CAU 2 or CAU 3 (92.7 and 95.0 p. 100).

TABLE 3

Fertilization rate after intrauterine insemination of epididymal spermatozoa or normal mating

TABLEAU 3

Pourcentage de fécondation après insémination intra-utérine avec des spermatozoïdes de l'épididyme ou après accouplement normal

Insemination	Number females	Number females with fertilized ova	Total number ova	Number fertilized ova	Percentage fertilization
Sperm suspension from :					
Caput epididymidis	7	1	47	1	2.1
Upper *corpus epididymidis*	7	0	46	0	0
Lower *corpus epididymidis*	13	12	142	81	57.0
Upper proximal *cauda epididymidis*	2	2	19	14	73.6
Proximal *cauda epididymidis*	11	9	83	77	92.7
Distal *cauda epididymidis*	26	26	162	154	95.0
Normal mating	35	35	335	319	95.2

Incidence of pronuclear ova with more than two pronuclei

Five pronuclear ova possessed three or four pronuclei for the whole series (table 4). Four were definitely trinucleate ; in the last one pronuclei were so close that it could not be determined with certainty if four pronuclei were present, but the outline of at

TABLE 4

Distribution of undivided ova with three or four pronuclei 24 to 29 hours post HCG injection

TABLEAU 4

Distribution d'œufs non divisés avec trois ou quatre pronuclei 20 à 24 heures après injection de HCG

Insemination	Total number undivided ova	Number of ova with three-four pronuclei	Percentage of ova with three-four pronuclei (%)
Sperm suspension from :			
Lower *corpus epididymidis*	27	3	11.1
Proximal *cauda epididymidis*	10	1	10.0
Distal *cauda epididymidis*	17	1	5.8
Normal mating	13	0	0

least three was quite definite. One ovum fertilized by spermatozoa from the junction COR 2, CAU 3 had only one pronucleus.

Embryonic mortality

Only 25 p. 100 of the females inseminated with spermatozoa from the COR 2 were pregnant when checked at day 10. The pregnancy rate in females inseminated with spermatozoa from the CAU 3 was very good (90 p. 100), but it became soon evident that in this group pre-implantation losses were quite heavy.

When spermatozoa from the COR 2 are inseminated, 82.1 p. 100 of the ova ovulated fail to implant (table 5). Since the fertilization rate is 57 p. 100, 39.1 p. 100 of the fertilized ova were lost between fertilization and implantation. When spermatozoa from the CAU 3 are inseminated, 60.4 p. 100 of the ovulated ova (55.4 p. 100 of the fertilized ova) degenerate before implantation. The same comparison shows that, when ova are fertilized with ejaculated spermatozoa surgically deposited in the uterus, 19.7 p. 100 of the fertilized ova are lost before implantation.

TABLE 5

Pre-implantation losses ten days after intrauterine insemination of epididymal or ejaculated spermatozoa

TABLEAU 5

Mortalité embryonnaire dix jours après insémination intra-utérine de spermatozoïdes de l'épididyme ou éjaculés

Insemination	Number of *corpora lutea vera*	Number of implantation sites	Size of implantation sites (mm)	Percentage of ovulated ova failing to implant	Percentage of fertilized ova failing to implant
Sperm suspension from :					
Lower *corpus epididymidis* .. ($6.9 \pm 1.0 \times 10^6$ sp./insem.)	128	23	14.30* ± 0.07**	82.1	39.1
Distal *cauda epididymidis*.... ($6.2 \pm 0.7 \times 10^6$ sp./insem.)	101	40	14.60 ± 0.30	60.4	55.4
Ejaculated semen..... ($6.0 \pm 0.4 \times 10^6$ sp./insem.)	90	68	14.43 ± 0.17	24.5	19.7

* Only uterine swellings with a diameter under 10 mm were considered as undergoing resorption and were not included in the calculations.

** Standard error.

As shown in Table 6, the respective percentages of implants failing to survive when spermatozoa from the COR 2, CAU 3, or ejaculate are used for insemination are 38.5, 10.0 and 19.2 p. 100. It would seem that the embryo loss is heavier when

spermatozoa from the COR 2 are inseminated. It should be noted that very few ova implant in the first case, and thus the percentage of post-implantation losses was determined on only 13 implantation sites, making this series hardly comparable to the others. Since the losses seem to occur before implantation, the pre-implantation period was further studied.

TABLE 6

Post-implantation losses between 10 and 28 days after intrauterine insemination of epididymal or ejaculated spermatozoa

TABLEAU 6

Mortalité embryonnaire entre le 10eme et le 28eme jours après insémination intra-utérine avec des spermatozoïdes de l'épididyme ou des spermatozoïdes éjaculés

Insemination	Number of *corpora lutea*	Number of fœtuses		Number of resorption sites	Litter size	Weight of normal live fœtuses	Percentage of implants failing to survive
		Live	Dead				
Sperm suspension from :							
Lower *corpus epididymidis*	116*	8	0	5	4.0	34.36 ± 0.95**	38.5
Distal *cauda epididymidis*	101	36	1	2	4.5	34.55 ± 0.95	10.0
Ejaculated semen.....	90	55	4	13	6.1	28.75 ± 0.81	19.2

* One female (10 implantation sites) died before day 28.
** Standard error.

Rate of segmentation

The normal rate of segmentation of ova was first established on 36 mated does killed 24 to 29 hours post HCG injection. Results are shown in table 7. The rate of segmentation of ova fertilized with epididymal spermatozoa was then studied.

In a first series, females previously mated or inseminated with a like number of spermatozoa from the COR 2 or the CAU 3 were killed 25 hours after HCG injection. For this group the segmentation rate of the ova is shown in table 8. After normal mating, 7.2 p. 100 of the fertilized ova recovered are still uncleaved and both pronuclei can be seen. When sperm suspensions from the CAU 3 are inseminated, 19.7 p. 100 of the fertilized ova are not segmented. This percentage rises to 51.3 when spermatozoa from the COR 2 are used ; in this case, no ova have yet passed the two-cell stage, while of the ova fertilized with CAU 3 or ejaculated spermatozoa, 4.2 and 3.6 p. 100 are already three or four-cell.

In a second series, does were kiled 27 hours post HCG injection. Results are

TABLE 7

Rate of segmentation of rabbit ova after double mating

TABLEAU 7

Vitesse de segmentation des œufs de lapin après double accouplement

	Interval HCG injection-autopsy (days)					
	24	25	26	27	28	29
Number of animals	6	6	6	6	6	6
Total number of ova	54	56	57	56	65	57
Number of unfertilized ova	3	1	4	1	1	6
Number of fertilized ova .	51	55	53	55	64	51
Percentage of undivided ova	11.7	7.2	3.7	1.8	0	0
Percentage of 2-cell ova .	86.2	89.0	60.3	20.0	50.0	15.6
Percentage of 3-cell ova .	0	1.8	3.7	10.9	1.5	11.7
Percentage of 4-cell ova .	1.9	1.8	32.0	67.2	48.4	72.5

TABLE 8

Segmentation of rabbit ova after intrauterine insemination of epididymal spermatozoa (25 hours post HCG injection)

TABLEAU 8

Segmentation des œufs de lapin après insémination intra-utérine avec des spermatozoïdes de l'épididyme

	Insemination with epididymal sperm suspension (1) from		Normal mating
	Lower *corpus epididymidis*	Distal *cauda epididymidis* (2)	
Number of animals.....	7	12	6
Total number of ova	67	81	56
Number of unfertilized ova ..	30	10	1
Number of fertilized ova.....	37	71	55
Percentage of undivided ova .	51.3	19.7	7.2
Percentage of 2-cell ova ...	48.6	76.0	89.0
Percentage of 3-cell ova ...	0	2.8	1.8
Percentage of 4-cell ova ...	0	1.4	1.8

(1) Number spermatozoa/inseminate = $4.7 \pm 1.1 \times 10^3$.

(2) Five does in this series were inseminated with 15.1×10^3 spermatozoa instead of 4.7×10^6.

shown in table 9. After normal mating, only 1.8 p. 100 of the fertilized ova are still undivided. When spermatozoa from the CAU 3 are inseminated, 5.2 p. 100 of the ova are unsegmented. This percentage rises to 18.1 when COR 2 spermatozoa are used ;

TABLE 9

Segmentation of rabbit ova after intrauterine insemination of epididymal spermatozoa (27 hours post HCG injection)

TABLEAU 9

Segmentation des œufs de lapin après insémination avec des spermatozoïdes de l'épididyme (27 heures après injection de HCG)

	Insemination with epididymal sperm suspension (1) from		Normal mating
	Lower *corpus epididymis*	Distal *cauda epidiaymidis*	
Number of animals.....	6	7	6
Total number of ova	75	57	58
Number of unfertilized ova	31	0	3
Number of fertilized ova...	44	57	55
Percentage of undivided ova .	18.1	5.2	1.8
Percentage of 2-cell ova ...	70.4	57.8	20.0
Percentage of 3-cell ova ...	0	3.5	10.9
Percentage of 4-cell ova ...	11.3	33.3	67.2

(1) Number spermatozoa/inseminate = 8.8×10^6.

in this group, 11.3 p. 100 of the ova have progressed beyond the two-cell stage. In the CAU 3 and the normal mating groups, 36.8 and 78.1 p. 100 of the ova are three or four-cell. These results clearly indicate that ova fertilized with spermatozoa from the COR 2 do not present the same stage of development at 25 or 27 hours post HCG injection as the ova fertilized by spermatozoa from the CAU 3 or after normal mating. There is a very significant difference between these two latter groups, indicating that even ova fertilized by spermatozoa from the CAU 3 are slightly delayed compared to the control.

DISCUSSION

It seems that spermatozoa from the *caput epididymidis* have a very small fertilizing capacity, if any. BLANDAU and RUMERY, 1964, report that, in the rat, only 8 p. 100 of the ova were fertilized when spermatozoa from the *caput epididymidis* were instillated in the *cornua*,, in contrast with 93 p. 100 when spermatozoa from the

cauda were used. In the rabbit, 16.6 p. 100 of the females inseminated vaginally with spermatozoa from the *caput epididymidis* gave birth (one young for 6 females), 25 p. 100 delivered after insemination with spermatozoa from the *corpus*, and 14.3 p. 100 and 50.3 p. 100 delivered after insemination of spermatozoa from either the proximal or the distal *cauda* (NISHIKAWA and WAIDE, 1952). More recently, BEDFORD (1966), reported that in rabbits the fertilizing ability was acquired by spermatozoa as they attained the middle *corpus epididymidis* : virtually no ova were fertilized after insemination into the oviduct of sperm suspensions from the *caput* or the upper *corpus epididymidis*, and 97 p. 100 were fertilized when spermatozoa from the lower *corpus* and the *cauda epididymidis* were used. Our results confirm these data but, using intrauterine insemination, our percentage of fertilized ova reaches 92.7 only when proximal *cauda* spermatozoa are used for insemination.

There is no difference in the percentage of no-droplet spermatozoa betveen the upper and lower *corpus epididymidis*. Furthermore, when does were inseminated with sperm suspensions from the *caput epididymidis*, the total number of spermatozoa present was 15.0×10^6. As shown in table 1, 2.5×10^6 of these spermatozoa were free of cytoplasmic droplets, and yet no fertilization occurs. When sperm suspensions from the upper *corpus*, containing 1.3×10^6 of no-droplet spermatozoa, were used, no fertilization was observed, although this number was sufficient to ensure fertilization (CHANG, 1946 ; AUSTIN, 1948). Thus, it does not seem that in the rabbit the absence of the cytoplasmic droplet can give an accurate index of sperm maturation in terms of fertilizing ability. In the rat, BLANDAU and RUMERY, 1964, attribute the lack of fertilization with spermatozoa from the *caput epididymidis* to their non-forward progression and thus to their inability to go through the utero-tubal junction. They found a small number of spermatozoa in only one of fifteen oviducts, 30 hours after insemination with sperm suspensions from the *caput epididymidis* ; but the comparative data for the control oviducts, where sperm suspensions from the *cauda* were inseminated, are not given. In the rabbit, we have observed that 73 p. 100 of spermatozoa from the *caput epididymidis* display an individual, circular type of movement described as « spinning », but that their motility was too weak to induce formation of circular waves as described by BLANDAU and RUMERY in the rat. Going down the epididymis toward the *ductus deferens*, the type of motility changes : 68 p. 100 of the spermatozoa from the *cauda* swim in large circular paths characterized by the non-rotation of the sperm head, and 31 p. 100 progress forward, rotating along their longitudinal axes and moving in fairly straight paths. Of the spermaozoa recovered 25 to 29 hours after insemination from the oviducts, all those still motile were progressing forward, regardless of the epididymal segment they came from (unpublished observations). Despite these differences in sperm motility, even spermatozoa from the *caput epididymidis* are able to pass through the utero-tubal junction, since $3\,900 \pm 1\,200$ can be found in the oviduct 27-28 hours after intrauterine insemination. It should be noted, however, that these figures do not indicate whether the spermatozoa were in sufficient numbers at the site of fertilization at the right moment. At any rate, when 15×10^6 spermatozoa from the *caput* are inseminated, approximately 1.6×10^6 are rotating cells progressing forward. This number is amply sufficient to ensure fertilization (CHANG, 1946 ; AUSTIN, 1948). The pattern of swimming movements is probably one facet of the very complex sperm maturation process, but is not by itself the sole factor responsible for attaining fertilizing ability.

Estimation of pre-implantation losses was based on the comparison of the number of *corpora lutea* in the ovaries observed at laparotomy at day 10 with the number of implantation sites in the uteri at the same time. The number of *Corpora lutea vera* was assumed to represent exactly the number of ova ovulated. *Corpora lutea atretica* amount to 4.2 p. 100 of the *corpora lutea vera*, an estimation very close to the one given by ADAMS (1960) for the domestic rabbit (3.8 p. 100). They were not included in the count mainly because of precedent (ALLEN, and *al.*, 1947; HALLIDAY, 1959; ADAMS, 1960), but no evidence is available as to whether they may represent ovulation or not. Although two or more ova may be liberated from one follicle, in our series the number of implantation sites never exceeded the number of *corpora lutea*: thus the possibility of polyovuly was not taken into account. The percentage of pre-implantation losses in our control series (24.5 p. 100) is higher than the one reported by BRAMBELL and MILLS, 1947, in wild rabbits (10.2 to 13.0 p. 100) or by ADAMS, 1960, in domestic rabbits (16.5 p. 100). The difference may be due to the additional stress caused by the surgical procedure used for insemination. The post-implantation losses were determined by the comparison of the number of implantation sites at day 10 of gestation with the number of surviving embryos at day 28. Nineteen percent of the embryos disappeared or died before the end of gestation in the control series. This is in close agreement with the data of ADAMS, 1960, in the domestic rabbit (20.2 p. 100). When the groups of does inseminated with epididymal spermatozoa are compared to the control, *it appears that as many as 77.6 and 65.4 p. 100 of all fertilized ova are lost when does are inseminated with spermatozoa from the lower* corpus epididymis *and the* distal cauda epididymidis *as compared to 38.9 p. 100 when ejaculated semen is used and that the losses occur before implantation rather than after.*

During the pre-implantation period the first obvious difference between ova fertilized with epididymal or ejaculated spermatozoa was the incidence of polyploid ova. Since does were killed 24 to 29 hours post HCG injection, most of the ova were divided and only 54 ova were still at the pronuclear stage. But 9.2 p. 100 of these ova had three of four pronuclei. It is not known if the same percentage of the divided ova presented the same abnormality. BEDFORD (1966) reports that 7 p. 100 and 5 p. 100 of the ova fertilized by spermatozoa from the *corpus* and *cauda* regions respectively, possesses three or four pronuclei. In rabbits normally mated or inseminated before ovulation, no polyploidy is observed, as in our control series (AUSTIN and BRADEN, 1953 ; AUSTIN, 1960 ; THIBAULT, 1967).

The second difference was the striking delay in the cleavage rate experienced by ova fertilized with epididymal spermatozoa. Ova fertilized by spermatozoa from the lower *corpus epididymidis* are more severely affected, but even ova fertilized by spermatozoa from the distal *cauda* are delayed as compared to the control. The cause of this altered cleavage rate is not clear at the present time. Epididymal spermatozoa may require a longer time to ascend into the oviduct through the utero-tubal junction or they may require a longer time for capacitation. In either case, the fertilization will be delayed, and spermatozoa will enter aged ova, a situation known to lead to polyploidy (AUSTIN, 1960 ; THIBAULT, 1967).

However, polyploidy can also be explained by the fact that immature spermatozoa may not be able fully to activate the ova. Instead of being abstricted, the chromatin of the second polar body remains in the ooplasm producing a triploid ovum. Polyploidy could, then, be considered the result of sperm immaturity. In the

present study no definite conclusion can be drawn as to the cause of the observed delayed segmentation and polyploidy since ova were recovered between 24 and 29 hours after HCG injection. To elucidate the problem, ova should be recovered from the time of fertilization until 24 hours after HCG.

To explain the embryonic mortality observed after insemination of epididymal spermatozoa, 2 hypotheses may be postulated : the first one is that the altered cleavage rate leads to an asynchronism in the stages of development of the ova and uterus. Under these conditions rabbit ova failed to implant (CHANG, 1950). The second hypothesis is that the embryonic deaths are related to the polyploid condition of some of the ova. These polyploid ova can segment normally at least up to the 8-cell stage without showing an, noticeable abnormalities (AUSTIN and BRADEN, (1953). In the rabbit, they can develop up to mid-gestation (BOMSEL-HELMREICH and THIBAULT, 1962) and losses grow progressively with advancing gestation (BOMSEL-HELMREICH, 1965, 1967). In our series, 9.2 p. 100 of the non-divided ova fertilized with epididymal spermatozoa are effectively polyploid. But it should be noted that nothing is known about the already-divided ones. Furthermore, in our series, pre-implantation losses are higher than post-implantation losses. whereas polyploidy should cause higher post-implantation losses. Further studies are necessary to clarify this point.

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SUMMARY

In this experiment, the ability of epididymal spermatozoa to fertilize and to produce normal viable embryos was studied. When surgically inseminated in the uterus of does injected with 50. IU of human chorionic gonadotrophins (APL-Ayers Laboratories) one hour previously, spermatozoa from the *caput* and the proximal *corpus epididymidis* were found infertile. In contrast, 57 per cent of 142 ova were fertilized in does inseminated with spermatozoa from the distal *corpus epididymidis*, 92.7 per cent of 83 ova and 95 per cent of 162 ova were fertilized when spermatozoa from the proximal and the distal *cauda epididymidis*, respectively, were used for insemination. Does were allowed to continue their pregnancy, then were laparotomized at day 10 to count the number of *corpora lutea* (ova ovulated) and the number of implantation sites, and were killed at day 28 to record the number of viable embryos. It was found that 30.1 per cent of the fertilized ova were lost before implantation and 38.5 per cent of the implants failed to survive when spermatozoa from the distal *corpus* were inseminated. Pre-implantation losses and post-implantation losses when distal *cauda* spermatozoa were inseminated were. 55.4 and 10 per cent respectively. When ejaculated spermatozoa were surgically inseminated in the uterus, percentages of losses were 19.7 and 19.2.

In an attempt to elucidate the cause of preimplantation losses when epididymal spermatozoa were used for insemination, ova were recovered 25 and 27 hours after HCG injection. It was found

that when distal *corpus* spermatozoa were used for insemination, fertilization was strikingly delayed : 25 hours after HCG 51.3 per cent of all ova are still undivided when spermatozoa from the lower corpus are used for insemination. Only 7.2 per cent are undivided 25 hours after mating. Twenty-seven hours after HCG, the respective percentages are 18.1 and 7.8. Fertilization of aged ova is known to lead to abnormality, such as polyploidy (AUSTIN, 1960 ; THIBAULT, 1967). Polyploid ova were indeed observed in the series : 10.2 per cent of 118 pronuclear ova exposed to epididymal spermatozoa possessed 3 or 4 pronuclei. No polyploidy was observed in 26 ova exposed to ejaculated spermatozoa. The possible reasons for both delayed fertilization and embryonic mortality is discussed.

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